Angiotensin II upregulates LDL receptor-related protein (LRP1) expression in the vascular wall: a new pro-atherogenic mechanism of hypertension

Judith Sendra1, Vicenta Llorente-Cortés1, Paula Costales1, Claudia Huesca-Gómez1, and Lina Badimon1,2*

1Barcelona Cardiovascular Research Center, CSIC-ICCC, Hospital de la Santa Creu i Sant Pau, Av. S. Antoni M. Claret 167, 08025 Barcelona, Spain; and 2CIBERONB, Instituto Salud Carlos III, Barcelona, Spain

Received 15 October 2007; revised 15 January 2008; accepted 4 February 2008; online publish-ahead-of-print 15 February 2008

Time for primary review: 17 days

Aims Hypertension is a risk factor for atherothrombotic vascular events. Angiotensin II (Ang II), one of the main vasoactive hormones of the renin–angiotensin system, has been associated with the development and progression of atherosclerosis. However, it is not fully known how Ang II contributes to lipid-enriched atherosclerotic lesion formation. In human vascular smooth muscle cells (VSMC), low density lipoprotein (LDL) receptor-related protein (LRP1) internalizes cholesteryl esters (CE) from extracellular matrix-bound aggregated LDL (agLDL). The aim of this study was to investigate the effect of Ang II on LRP1 expression and function in VSMC.

Methods and results Here, we report for the first time that Ang II induces the upregulation of LRP1 expression in VSMC. Ang II (1 μM) induced maximal LRP1 mRNA expression at 12 h and maximal protein overexpression (by 4.10-fold) at 24 h in cultured human VSMC. Ang II effects were functionally translated into an increased CE accumulation from agLDL uptake (by two-fold at 50 μg/mL) that was prevented by the LRP1 ligand lactoferrin and by siRNA-LRP1 treatment. Ang II-LRP1 upregulation and excess CE accumulation from agLDL were prevented by losartan (an AT1 blocker) but not by PD123319 (a specific AT2 blocker). Additionally, in a normolipidaemic rat model, Ang II infusion produced a significant increase in aortic LRP1 expression and lipid infiltration in the arterial intima.

Conclusion The in vitro and in vivo data reported here indicate that Ang II upregulates LRP1 receptor expression and LRP1-mediated aggregated LDL uptake in vascular cells.

KEYWORDS
Angiotensin II; Aggregated LDL; LRP1; AT1 receptors; Vascular smooth muscle cells; Arteriosclerosis

1. Introduction
Angiotensin II (Ang II), the primary vasoactive hormone in the renin–angiotensin system (RAS), initiates cellular responses by activation of type I (AT1) and type 2 (AT2) angiotensin receptors.1,2 Several clinical studies suggest that Ang II is involved in the development of atherogenesis and that inhibition of Ang II interaction with vascular cells attenuates the atherosclerotic process.3–8 One of the main events in vascular remodelling and atherosclerotic lesion progression is the accumulation of lipid in the arterial wall. Exposure to Ang II has been shown to induce atherosclerotic lipid infiltration in the aortas of apoE-deficient mice without changes in lipid levels.9 However, whether this effect is haemodynamic or receptor-mediated is unknown. LRP1 is overexpressed in advanced lipid-enriched atherosclerotic plaques in several human and animal models,10–13 and experimentally induced hypercholesterolemia increases LRP1 expression in the vascular wall.14 Recently, we have also demonstrated that LRP1-mediated agLDL uptake contributes to (i) the prothrombotic transformation of vascular smooth muscle cells (VSMC) by increasing membrane Tissue Factor (TF) activation and TF-enriched microparticle release; and (ii) to a distinctive pattern of VSMC-migration kinetics and remodelling.15–18

We hypothesized that Ang II might upregulate LRP1 in the vascular wall contributing to cell mediated vascular remodelling and lipid processing in the vascular wall. Therefore, the aim of this study was to investigate the effect of Ang II on LRP1 expression and function and to determine the Ang II receptor involved in this process. Our results demonstrate that Ang II upregulates LRP1 mRNA and protein expression. Ang II-induced LRP1 upregulation in human VSMC leads to an increased CE accumulation that is prevented by lactoferrin, an LRP1 ligand and by LRP1 silencing. Losartan (an AT1 blocker) but not by PD123319 (an AT2 blocker) prevent Ang II induced LRP1 overexpression and CE accumulation in VSMC.

* Corresponding author. Tel: +34 935655880; fax: +34 935655559.
E-mail address: lbadimon@csic-iccc.santpau.es

Published on behalf of the European Society of Cardiology. All rights reserved. © The Author 2008.
For permissions please email: journals.permissions@oxfordjournals.org.
human VSMC. Additionally, Ang II-infused animals showed LRP1 overexpression in the vascular wall. Thus, Ang II-induced LRP1 overexpression might be a pivotal mechanism promoting VSMC mediated remodelling, lipid accumulation, and atherosclerosis in hypertension.

2. Methods

2.1 Materials

Cell culture medium (M199) and reagents were provided by Gibco laboratories. The bicinechonic acid assay was from Pierce. The agarose gel electrophoresis Paragon System and Limulus Amebocyte Lysate endotoxin test were from Beckmann and Bio Whittaker, respectively. Tripure TM Isolation Reagent was from Roche Molecular Biochemicals. Real-time PCR reagents were from PE Biosystems. Anti-β chain LRP1 receptor antibodies were from Research Diagnostics (clone BBB RDI 61067); anti-AT1 and anti-AT2 receptors were from Santa Cruz (sc-1173 and sc-7421, respectively); anti-α-actinin antibodies were from Chemicon International (MAB 1682). Ang II (Sigma A9525), bovine lactoferrin (Sigma L4894), and PD123319 (Sigma P-186) were from Sigma Aldrich. Losartan was kindly provided by Merck Sharp Dhome.

2.2 VSMC culture

Primary cultures of human VSMC were obtained from non-atherosclerotic areas of human coronary arteries of hearts explanted from patients undergoing heart transplantation at the Hospital de la Santa Creu i Sant Pau, as previously described.13–16 The study was approved by the Reviewer Institutional Committee on Human Research of the Hospital of Santa Creu i Sant Pau that confirms to the Declaration of Helsinki. Arrested VSMC were pre-incubated with Ang II (1 μmol/L) for increasing periods of time. In some experiments, VSMC were pre-incubated with losartan (an AT1 receptor antagonist) (10 μmol/L) or PD123319 (an AT2 antagonist) (10 μmol/L) for 3 h before VSMC exposure to Ang II for 18 h. To analyse the effect of Ang II on the capacity of LRP1 to internalize agLDL, VSMC were incubated with Ang II for 18 h and then exposed to increasing concentrations of agLDL (0, 25, 50, and 100 μg/mL) for 6 h. To analyse the involvement of LRP1 on Ang II-induced CE accumulation, VSMC were pre-incubated with lactoferrin (an LRP1 ligand; 19,20 25 or 50 μg/mL) for 2 h before adding agLDL (50 μg/mL). Ang II, lactoferrin, losartan or PD123319 did not produce any effect on cell morphology or cell apoptosis (assessed by staining with Trypan Blue).

2.3 Treatment of VSMC with siRNA-LRP1

In order to inhibit LRP1 expression in VSMC (siRNA-LRP1-VSMC), human cells were transiently transfected with annealed siRNA. LRP1 specific siRNA was synthesized by Ambion according to our previously published LRP1 target sequences.21–23 Sense and random oligodeoxy nucleotides did not exert any effect on LRP1 expression.21 Fasta analysis (Genetic Computer Group Package) indicated that these sequences would not hybridize to other receptor sequences (including LDL receptor) in the GenBank database. In agreement, siRNA-LRP1 treatment did not alter LDLR expression13 or native LDL uptake by vascular cells.21 In brief, VSMC were transfected with siRNA-LRP1 (50 nM) using siPORT NeoFx in serum-free DMEM medium (1% glutamine) according to the kit instructions (SilencerTM siRNA Transfection Kit; Ambion no. 4511). This medium with siRNA-LRP1 was maintained for 36 h and it was then replaced by a new medium containing Ang II (1 μM). After 18 h, agLDL (50 μg/mL) was added to the medium and maintained for 6 h. Cells were then exhaustively washed and harvested to test LRP1 expression. Extra wells were used in order to test the specificity of siRNA-LRP1 treatment by analysing LDLR mRNA expression by real-time PCR. The cells did not take up Trypan Blue, and their morphology was not altered by the procedure.

2.4 Animals

The in vivo effects of Ang II were evaluated in male Wistar rats subcutaneously implanted with osmotic minipumps (Alza corp., Palo Alto, CA, USA) that infused saline (Group I, n = 6), Ang II at 50 ng/kg/min (Group II, n = 3), or Ang II at 200 ng/kg/min (Group III, n = 5) for 14 days. Systolic blood pressure (SBP) measurements were performed in conscious, restrained rats by tail-cuff plethysmography (ML125, Power Lab) at day 1, 3, 5, 7, 10, and 13 post-osmotic pump implantation. On day 14, animals were euthanized and aortas dissected. One portion was immediately prepared for histological studies. Another portion was frozen in liquid nitrogen and kept at −80°C. One aliquot of this portion was used for mRNA and protein extraction and other aliquot for lipid extraction. Lipids and biochemical parameters were measured by an automatic Analyzer (RAL Clima MC). All procedures were in accordance with institutional guidelines and confirm with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

2.5 LDL isolation and modification

Human LDL (d₁₀₁₀₀, 1.019 g/mL) were obtained from pooled sera of normcholesterolemic volunteers by sequential ultracentrifugation. AgLDL were prepared as previously described 13–16. TBARS levels (<1.2 mmol malonaldehyde per milligram of protein LDL) remained similar to those in nLDL after LDL aggregation.

2.6 Determination of free and cholesteryl ester content

Control and Ang II treated VSMC (1 μM, 18 h) were incubated with increasing concentrations of agLDL (0, 25, 50, and 100 μg/mL) for 6 h. Following the lipoprotein incubation period, cells were exhaustively washed, and lipid extraction was performed as previously described.13–16 One portion of the aorta frozen in liquid nitrogen (25 mg) was also homogenized and lipids extracted.13,14 The spots corresponding to free cholesterol (FC) and CE in the thin layer chromatography were quantified by densitometry against the standard curve of cholesterol and cholesteral palmitate, respectively, using a computing densitometer (Molecular Dynamics).

2.7 Real-time PCR

Total RNA and protein were isolated using the Tripure™ Isolation Reagent (Roche Molecular Biochemicals) according to the manufacturer’s instructions. LRP1, AT1, and AT2 mRNA levels were analysed by using real-time PCR. TaqMan fluorescent real-time PCR primers and probes (FAM-MGB) for LRP1, AT1, and AT2 were designed by the use of Primer Express software from PE biosystems, and were as follows: LRP1 forward, 5'-gtggaacagccacgctttg-3'; LRP1 reverse, 5'-ggtagaacatgcacctgcctatc-3'; LRP1 Probe, 5'-ttgcttcggtgc acag-3'; AT1 forward, 5'-atgctgacagcagtaaaa-3'; AT1 reverse, 5'-tctgacctgagacctttg-3'; AT1 Probe, 5'-ttgcttcggtgcctaa-3'; AT2 forward, 5'-caacctcactgcctacta-3'; AT2 reverse, 5'-ggtagttcacaagccgcta-3'; AT2 probe, 5'-acacattacacctg-3'. Rat LRP1 was detected by a Custom TaqMan Assay (Applied Biosystems). Human gapdh (4326317E) was used as endogenous control in human VSMC and 18 sRNA was used as an endogenous control in rats. Taqman real-time PCR was performed as previously described.14

2.8 Western-blot analysis

Proteins were analysed by western-blot analysis as previously described.14 Blots were incubated with monoclonal antibodies against human LRP1 (β-chain, clone 88B RDI 61067, dilution 1:40) or polyclonal antibodies against AT1 (Santa Cruz, N-10, dilution 1:200) or AT2 (Santa Cruz, N-19, dilution 1:100) receptor. Equal loading of protein in each lane was verified by staining filters with
2.9 Immunofluorescence labelling and confocal microscopy

To analyse the effect of Ang II on the capacity of VSMC to internalize agLDL, cells were incubated with Ang II for 18 h and then exposed to agLDL (50 μg/mL) for 6 h. Cells were then exhaustively washed and stained with DiI (30 μg/mL). After 30 min at 37°C, cells were washed and fixed with 3.5% paraformaldehyde, permeabilized with 0.5% Tween–PBS, and blocked with 1% BSA in 0.1% Tween–PBS. Nuclei were stained with Hoechst (Molecular Probes). Immunolabeled cells were examined in an inverted fluorescence confocal microscope (Leica TCS SP2-AOB) and processed with the TCS-AOBS software (Leica).

2.10 Immunohistochemistry

Rat aorta arteries were dissected and immersed in 4% paraformaldehyde fixative solution, sectioned into blocks, cryoprotected with saccarose (30%), included in OCT, and frozen. Vessels were transversely cut and consecutive sections (5 μm thick) were collected on chromopotentassium-gelatin-coated slides for immunohistochemistry (stored at −20°C). Sections were thawed, the endogenous peroxidase activity was blocked with H2O2 (in methanol for 30 min), and non-specific blocking was performed with PBS-10% horse serum. Masson–Biebrich Trichromic staining was performed to visualize the arterial wall morphology. Lipid staining was performed with Herxheimer reagent. Tissue sections were treated with monoclonal antibody to LRP1 antibodies (clone 5A6, RDI 61066), to smooth muscle cell actin (α-SMC; Dako M0851) or to HAMS 56 (Dako M0632) for 18 h at 4°C. Biotinylated anti-mouse IgG (Vector) was used as a secondary antibody. The primary antibody was detected using the Avidin–Biotin immunoperoxidase technique, and the chromogen was DAB. Normal non-immune serum was substituted for primary antibody as a negative control. Images were captured with a Nikon Eclipse 80i microscope, and digitized by a Retiga 1300i camera.

2.11 Data analysis

Data were expressed as mean ± SEM. A statview (Abacus Concepts) was used for statistical analysis. Multiple groups were compared by non-parametric tests, Mann–Whitney U or Kruskal–Wallis tests as needed. Statistical significance was considered when P < 0.05.

3. Results

3.1 Angiotensin II induces LRP1 expression in human vascular smooth muscle cells

In human VSMC, Ang II induced LRP1 mRNA expression within a maximum of 12 h (by 1.76-fold; P < 0.05) (Figure 1A). Concomitantly, Ang II induced AT1 receptor mRNA expression (by 1.46-fold; P < 0.05) (Figure 1B). On the contrary, AT2 mRNA expression was not significantly altered by Ang II at
any tested time (data not shown). In agreement, LRP1 protein expression was upregulated by Ang II in a time-dependent manner from 1.68-fold at 2 h to 4.10-fold at 24 h (Figure 1C).

3.2 Angiotensin II induces CE accumulation derived from LRP1-mediated agLDL uptake in human VSMC

To investigate the effect of Ang II-mediated LRP1 induction on cell functionality, VSMC were incubated with Ang II for 18 h and then for 6 h with increasing concentrations of agLDL (0, 25, 50, and 100 μg/mL). As shown in Figure 2A, Ang II increased CE accumulation derived from agLDL at each tested concentration (from 53 ± 0.65 to 107 ± 2.2 μg CE/mg protein at 50 μg/mL and from 77.1 ± 02.1 to 135 ± 3.1 μg CE/mg protein at 100 μg/mL). Ang II did not significantly alter the FC content of VSMC exposed to agLDL. Accordingly, intracellular lipid staining was higher in Ang II-treated VSMCs compared to control VSMC, as evidenced by fluorescence confocal microscopy (Figure 2B).

3.3 Inhibition of LRP1 prevents agLDL-cholesteryl ester accumulation induced by Ang II

As shown in Figure 3A, the increase in agLDL-CE accumulation induced by Ang II was inhibited by lactoferrin (LRP1 ligand).19,20 Lactoferrin reduced CE accumulation derived from agLDL uptake from 91.4 ± 0.3 to 40.08 ± 0.24 μgCE/mg protein.

As previously demonstrated,21–23 siRNA-LRP1 treatment reduced LRP1 mRNA expression by approximately 73% in VSMC (data not shown). In agreement, siRNA-LRP1 treatment reduced Ang II induced CE accumulation derived from agLDL uptake from 90.0 ± 4.2 to 35 ± 3.2 μgCE/mg protein (Figure 3B). No effect of lactoferrin or siRNA-LRP1 treatment on intracellular FC content was observed. Taken together, these results demonstrate that the effect of Ang II on agLDL internalization is dependent on LRP1 function.

3.4 Angiotensin II induces LRP1 expression and function through AT1 receptor

Ang II induced LRP1 protein overexpression was completely prevented by the AT1 antagonist losartan (10 μmol/mL) but not by the AT2 antagonist PD123319 (10 μmol/mL) (Figure 4A). Losartan and PD123319 by themselves did not exert any significant effect on LRP1 protein expression. As shown in Figure 4B, the effect of Ang II on intracellular CE accumulation from agLDL uptake was blocked by losartan but not by PD123319. Losartan and PD123319 by themselves did not exert any significant effect on CE accumulation from agLDL uptake. No alterations on FC content of VSMC incubated with agLDL were observed by exposure to Ang II, losartan, or PD123315.
3.5 LRP1 expression levels were higher in the vascular wall of Ang II-infused rats

To determine whether Ang II influences the LRP1 expression in the vascular wall in vivo, normal rats were infused with Ang II. As shown in Table 1, no significant differences in metabolic, renal, or hepatic markers were found between saline and Ang II-infused animals. Cholesterol plasma levels were not significantly altered by Ang II infusion (Table 1), as expected from the animal model chosen. Indeed the proof of concept of Ang II effects on LRP1 required a normolipidaemic animal model because we have previously demonstrated that plasma cholesterol levels upregulate LRP1 expression levels in the vascular wall.14 SBP was dose-dependently increased by Ang II-infusion reaching significance at sacrifice (day 13) in group II and at day 5, 7, 10 and at sacrifice (day 13) in group III (Figure 5A). There was a significant positive correlation between LRP1 mRNA expression and SBP (at sacrifice day) in both thoracic ($r^2 = 0.66; P = 0.002$) (Figure 5B) and abdominal aorta ($r^2 = 0.55; P = 0.014$). Real-time PCR results demonstrate that the LRP1 mRNA expression was significantly upregulated both in the thoracic and abdominal aorta (Figure 6A) of Ang II-infused rats. LRP1 protein was equally significantly overexpressed in the thoracic and abdominal aorta of Ang II-infused rats from group III (Figure 6B).
only significantly upregulated in one of the three tested animals.

Interestingly, Ang II infusion increased CE accumulation in the aorta of Ang II 200 ng/kg min treated normocholesterolemic animals vs. placebo (44.7 ± 2.69 mg CE/gr aorta vs. 10.46 ± 0.1 mg CE/gr aorta, respectively) without altering the aortic FC content (25.2 ± 0.55 mg FC/gr aorta vs. 28.2 ± 0.81 mg FC/gr aorta, respectively) (Table 2).

Immunohistochemical analysis revealed overexpression of LRP1 in VSMC of animals treated with 200 ng/kg min for 14 days (Figure 7E). Monocyte/macrophage infiltration was rarely seen in the vascular wall of these animals (Figure 7I).

4. Discussion

Hypertension is a major risk factor for coronary and cerebrovascular diseases. In addition, hypertensive patients often have high cholesterol levels and an increased risk of atherosclerotic disease progression. However, the mechanisms by which the association of risk factors increases atherosclerosis are not clear. Recent studies have reported on the deleterious effect on the vascular wall of continuous exposure to Ang II.4–8 Our results show that in human VSMC, Ang II increases the expression of LRP1, a lipoprotein receptor that actively transforms VSMC in lipid-enriched foam cells.21–23 Here, we demonstrate that Ang II in the absence of LDL induces LRP1 mRNA expression and protein synthesis in human vascular cells. This upregulation of LRP1 has functional effects because it leads to intracellular CE accumulation from agLDL uptake.

Ang II induced intracellular CE overaccumulation was completely prevented by losartan (an AT1 antagonist) and lactoferrin (an LRP1 ligand).19,20 In agreement, Ang II does not induce CE overaccumulation in siRNA-LRP1–VSMC suggesting that the upregulatory effect of Ang II on LRP1 and LRP1-mediated agLDL uptake depends on functional AT1 and LRP1 receptors. In fact, in our cells AT1 and LRP1 were concomitantly upregulated by Ang II, in agreement with the positive modulation of AT1 receptors by Ang II.24–27 Indeed, Ang II is a vasoactive hormone that has been shown to
induce additional lipoprotein receptors in VSMC, like the lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1).

Interestingly, Ang II induces LRP1 in human VSMC to the same extent that agLDL, one of the most important LDL modifications in the arterial intima. These results suggest that several of the Ang II effects in vascular disease and atherosclerosis might be the consequence of its capacity to increase VSMC–foam cell formation. Most of the previous studies that analyse effects of Ang II on the vascular wall used apoE deficient-hypercholesterolemic mice with abnormally high plasma cholesterol levels. In these animals, Ang II complicates advanced atherosclerotic lesions by increasing the content of lipid and lipid-laden macrophages. In this study, we selected an experimental model with normal plasma cholesterol levels to dissect and analyse the effect of Ang II per se, separated from that of LDL, on vascular LRP1 expression. Immunohistochemical analysis of the aortas revealed that, as we expected in normal rats with average 65 mg/dL cholesterol plasma levels, there were not atherosclerotic lesions, macrophage infiltration, or lipid deposition in the treated or control animals. Because LRP1 is upregulated by high plasma cholesterol levels, an animal model with elevation of both risk factors would have not provided the adequate proof of concept. As observed in human VSMC in culture, Ang II induced the upregulation of LRP1 expression in the aorta of the rats in a dose-dependent manner. Interestingly, thin layer chromatography analysis of the aorta from Ang II-infused animals demonstrated that Ang II alone, without

**Table 2** Effect of angiotensin II on the cholesterol content of aorta

<table>
<thead>
<tr>
<th>Group</th>
<th>Free cholesterol</th>
<th>Cholesteryl esters</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>28.1 ± 0.81</td>
<td>10.46 ± 0.10</td>
</tr>
<tr>
<td>Ang 200</td>
<td>25.2 ± 0.55</td>
<td>44.7 ± 2.69*</td>
</tr>
</tbody>
</table>

Rats were administered saline buffer (n = 6) or Ang II at 200 ng/kg min for 2 weeks. Aortas were dissected, lipids extracted and analysed by thin layer chromatography. Results are expressed as micrograms of cholesterol per gram of aorta and are shown as mean ± SEM.

*P < 0.05, vs. saline.
hypercholesterolemia, slightly but significantly increased the cholesteryl ester content of the aorta. Ang II induced an increase in SBP that significantly correlates with LRP1 expression in the vascular wall. One of the mechanisms involved in this positive correlation is related to the effect of Ang II on LRP1 as demonstrated in cell culture. However, the small but significant increase in CE content in the aortas of Ang II-infused animals may be due to an increased influx of LDL cholesterol to the arterial intima. In fact, an increased transvascular LDL transport to the intima has been previously described in association with systolic hypertension in diabetes.31 Therefore, the LRP1 overexpression detected in the vascular wall of Ang II-infused rats might be caused not only by a direct effect of Ang II on LRP1 as demonstrated in cultured human VSMC but also by the upregulatory effect of intravascular LDL on LRP1 expression, as previously demonstrated.14 LRP1 plays an important role in maintaining the vascular integrity32 but LRP1 overexpression is also associated with cardiovascular risk factor induction of atherosclerotic lesion progression.14–18 Similarly, disregulation of a physiological haemostatic process can lead to a pathological thrombotic episode.

In summary, the in vitro human VSMC culture results reported here indicate for the first time that Ang II upregulates LRP1 receptor expression and LRP1-mediated agLDL uptake in vascular cells. These results are confirmed in vivo, in a rodent model where Ang II induced hypertension upregulates LRP1 expression. Ang II-induced VSMC–LRP1 upregulation may have an important role in the atherosclerotic lesion formation and deserves further investigation in additional models because it may have important clinical implications.

Acknowledgements
The authors thank the Heart Transplant Team of the Division of Cardiology and Cardiac Surgery at Hospital Santa Creu i Sant Pau and the Blood Bank at Hospital Vall d’Hebron, Barcelona, for their collaboration. The authors are indebted to O. Juan-Babot by his knowledge and technical practice in immunocytochemistry and to Sonia Sanchez, Albert Llenas, and Javier Blanco for their help with animal procedures. The authors also thank Laura Nasarre, Vanessa Martin, and Monica Carabias for their technical support.

Conflict of interest: none declared.

Funding
This work has been possible thanks to funding from FIS CIBER ONOB03/2006, REDINSCOR RD06/0003/0015 and FIS PI051717 from Instituto Salud Carlos III; SAF 2006/10009, and MSD-Unrestricted grant.
References


